ESTABLISHMENT OF A ZEBRAFISH EMBRYONIC STEM CELL LINE USING A FEEDER-FREE CULTURE METHOD AND *IN VITRO* PLURIPOTENCY CHARACTERIZATION

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by

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LIST OF ABBREVIATIONS

%	percentage
±	plus-minus
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
2D	two-dimensional
3D	three-dimensional
AP	alkaline phosphatase
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cm	centimeter
CMV	cytomegalovirus
c-Myc	v-myc myelocytomatosis viral oncogene homolog
CXCR4	C-X-C chemokine receptor type 4
DAPI	2-(4-amidinophenyl)-1H -indole-6-carboxamidine
dGH	degrees of general hardness

DMEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EB	embryoid bodies
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EPC	carp cell line
ES	embryonic stem
ESM2	ES medium 2
ESM4	ES medium 4
et al.	*et alii* (and other people)
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
Fbx15	*F-box protein 15*
Fgf-4	*fibroblast growth factor 4*
FITC	fluorescein isothiocyanate
FOXA2/HNF3β	forkhead box protein A2/ hepatocyte nuclear factor 3-beta
g	gram

g *relative centrifugal force*

G_1 growth 1

G418 geneticin

GBX2 gastrulation brain homeobox 2

GFP green fluorescent protein

gp130 glycoprotein 130

GSC goosecoid homeobox

GSK3 glycogen synthase kinase 3

HEPES 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

hES human embryonic stem

HMG high mobility group

hpf hours post-fertilization

ICM inner cell mass

IgG immunoglobulin G

iPS induced pluripotent stem

iSCNT interspecies somatic cell nuclear transfer

KCl potassium chloride

Klf4 kruppel-like factor 4

L liter

L-15	leibovitz's L-15
LDF	leibovitz L-15, dulbecco's modified eagle medium and ham's F-12
Lefty1	*left-right determination factor 1*
LIF	leukemia inhibitory factor
M	molar
MAP2	microtubule associated protein 2
MEF	mouse embryonic fibroblast
MEK-1	mitogen-activated protein kinase 1
mES	mouse embryonic stem
MES1	medakafish embryonic stem cell line
mitf	*microphtalmia transcription factor*
ml	milliliter
mm	millimeter
mM	millimolar
mstn 1	*myostatin 1*
NaClO	sodium hypochlorite
NaOH	sodium hydroxide
ng	nanogram

nM	nanomolar
ntl	*no tail*
^{o}C	degree Celsius
Oct-3/4	octamer-binding transcription factor 3/4
Oct4	octamer-binding transcription factor 4
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH	potentiometric hydrogen ion concentration
PNS	positive-negative selection
POU	derived from these three transcription factors: **p**ituitary-specific pit-1, **o**ctamer transcription factor proteins Oct-1 and Oct-2 and **U**nc-86 transcription factor from *C. elegans*
pou2	*pou class 2*
POU5F1	POU class 5 homeobox 1
POUV	POU class 5
RA	all-*trans*-retinoic acid
Rex-1	reduced expression 1
RFP	red fluorescent protein

rpm	revolutions per minute
RTS34st	rainbow trout spleen stromal cell line
Sall4	sal-like protein 4
SCNT	somatic cell nuclear transfer
SOX	SRY box-containing
SOX17	SRY (sex determining region Y)-box 17
SOX2	SRY (sex determining region Y)-box 2
SRY	sex determining region Y
T_D	population doubling time
Terc	telomerase RNA component
Tert	telomerase reverse transcriptase
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
U	units
UTF1	undifferentiated embryonic cell transcription factor 1
v/v	volume per volume
w/v	weight per volume
ZEB2J	zebrafish blastula cell line
ZES1	zebrafish embryonic stem cell-like line 1

LIST OF PUBLICATION

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PENGHASILAN TITISAN SEL TUNJANG EMBRIONIK DARIPADA IKAN ZEBRA DENGAN MENGGUNAKAN KAEDAH KULTUR TANPA LAPISAN SEL PENYUAP DAN PENCIRIAN PLURIPOTENSI *IN VITRO*

ABSTRAK

Lapisan sel penyuap telah biasa digunakan dalam kultur sel tunjang embrionik daripada ikan zebra untuk membolehkan proliferasi dan mencegah pembezaan sel-sel tersebut. Dalam kajian ini, kaedah kultur tanpa lapisan sel penyuap telah diguna untuk menghasilkan titisan sel tunjang embrionik jangka masa panjang daripada embrio peringkat blastula ikan zebra. Titisan sel ini dinamakan sebagai ZES1. Ia telah dikultur lebih daripada 800 hari dalam media asas, DMEM, ditambah dengan serum janin lembu, ekstrak embrio ikan zebra, serum ikan trout dan faktor pertumbuhan fibroblas asasi manusia. Sel ZES1 berbentuk bulat atau poligon dengan nukleus yang besar dan sitoplasma yang jarang. Majoriti sel ZES1 juga mempunyai kariotip diploid. ZES1 dapat membentuk koloni padat yang positif kepada fosfatase alkali dan membentuk badan embrioid apabila dikultur secara suspensi. Pluripotensi ZES1 telah disahkan apabila ia dapat membeza kepada beberapa jenis sel daripada tiga lapisan germa *in vitro*. Sel ZES1 yang dirawat dengan asid semua-*trans*-retinoik juga dapat membeza terutamanya kepada sel neuron. Semua jenis sel berbeza yang diperoleh daripada pembezaan ketumpatan tinggi dan rawatan asid semua-*trans*-retinoik telah dikenal pasti melalui immunokimia dengan menggunakan penanda protein khusus kepada sel keturunan. Immunokimia dengan menggunakan penanda protein berkaitan pluripotensi menunjukkan bahawa sel ZES1 adalah positif kepada SOX2 dan POU5F1 yang memainkan peranan penting dalam pembentukan awal embrio, walaupun POU5F1 juga dikesan dalam sel yang berbeza. Kesimpulannya, sel ZES1 memiliki ciri-ciri

pluripotensi *in vitro* dan boleh dikultur untuk jangka masa yang panjang dengan menggunakan kaedah kultur tanpa lapisan sel penyuap.

ESTABLISHMENT OF A ZEBRAFISH EMBRYONIC STEM CELL LINE USING A FEEDER-FREE CULTURE METHOD AND *IN VITRO* PLURIPOTENCY CHARACTERIZATION

ABSTRACT

Feeder layers are commonly used in zebrafish embryonic stem (ES) cell cultures to enable proliferation and prevent differentiation of the ES cells. In this study, a culture method using feeder-free was employed to derive and establish long-term zebrafish ES cell line from zebrafish blastula stage embryos. This cell line was designated as ZES1. It had been maintained for over 800 days in basal medium, DMEM, supplemented with fetal bovine serum, zebrafish embryo extract, trout serum and human basic fibroblast growth factor. ZES1 cells were round or polygonal in shape with large nuclei and sparse cytoplasm. Majority of ZES1 cells also displayed a diploid karyotype, typical of ES cells. ZES1 was able to form individual colonies of tightly packed cells that were positively stained for alkaline phosphatase and formed embryoid bodies when cultured in suspension. Pluripotency of ZES1 was confirmed when they were induced to differentiate *in vitro* at different seeding densities into several cell types from the three germ layers. ZES1 cells treated with all-*trans*-retinoic acid (RA) were also induced to differentiate into primarily neuronal cells. All the differentiated cell types obtained from high density differentiation as well as RA treatment were further identified through immunocytochemistry with cell lineage-specific protein markers. Immunocytochemistry using pluripotency-related protein markers showed that ZES1 cells were positively stained for SOX2 and POU5F1 that played critical roles during early embryogenesis, even though POU5F1 was also detected in differentiated cells. In conclusion, ZES1 cells possessed *in vitro*

pluripotency characteristics which can be readily derived and maintained for long-term under feeder-free culture conditions.

CHAPTER 1

INTRODUCTION

1.1 Research background

The extraordinary potentials of stem cells have brought on major breakthroughs for cell and developmental biology. Among the major breakthroughs are establishment of mouse embryonic stem (mES) cells (Evans & Kaufman, 1981; Martin, 1981), establishment of human embryonic stem (hES) cells (Thomson *et al.*, 1998), formation of mouse genetic models of disease by homologous recombination of ES cells (Thompson *et al.*, 1989), *in vitro* germ-line development of ES cells (Geijsen *et al.*, 2003; Hübner *et al.*, 2003; Toyooka *et al.*, 2003), as well as production of reprogrammed somatic cells following nuclear transfer into enucleated eggs (Wilmut *et al.*, 1997). All these breakthroughs have greatly benefited self-renewal studies in ES cells, cell therapies and regenerative medicine.

Attempts to establish ES cell cultures from other mammalian and non-mammalian species begin after the successful establishment of mouse and human ES cell lines (Yi *et al.*, 2010b). Initial efforts on deriving ES cell cultures from two small laboratory fish, medakafish (*Oryzias latipes*) and zebrafish (*Danio rerio*) have begun about 20 years ago (Yi *et al.*, 2010b). Efforts to establish ES cell lines from medakafish (Wakamatsu *et al.*, 1994) and zebrafish (Collodi *et al.*, 1992; Sun *et al.*, 1995a) have only met with partial success by using growth-arrested rainbow trout spleen stromal cell line, RTS34st or zebrafish embryonic fibroblasts in feeder layer culture methods (Alvarez *et al.*, 2007). Even though feeder layers provide factors and environment to support proliferation of pluripotent ES cells, they still remain poorly

defined and call upon the need to separate ES cell populations from feeder cells in order to study ES cells' characteristics precisely. Consecutively, feeder-free culture conditions were developed by Hong and Schartl, (1996) in medakafish ES cell cultures. This achievement has led to the establishment of several fish ES cell lines in gilthead sea bream (Béjar *et al*., 1999), red sea bream (Chen *et al*., 2003a) and sea perch (Chen *et al*., 2003b).

However, long-term culture of zebrafish ES cells in feeder-free culture conditions has not been reported. It was found that zebrafish embryo cells underwent extensive differentiations (Ma *et al*., 2001), had limited differentiation potential (Xing *et al*., 2008) and could only be maintained as transient cultures (Robles *et al*., 2011) when they were cultured in the absence of feeder layers. Among the challenges of deriving a feeder-free zebrafish ES cell line are prevention of extensive spontaneous differentiations in primary cultures and establishment of a long-term zebrafish ES cell line for further downstream experiments. In this study, the *in vitro* pluripotency and differentiation potential of a zebrafish ES cell line established under feeder-free culture method will be examined.

1.2 Objectives

In order to develop a long-term zebrafish ES cell culture method, zebrafish embryos will be utilized for the establishment of ES cells under feeder-free conditions. The major objectives of this study are:

I. To establish a long-term and stable embryonic stem cell-like line from zebrafish blastula stage embryos in a feeder-free culture system.

II. To carry out *in vitro* characterizations of the derived zebrafish embryonic stem cell-like line.

CHAPTER 2

LITERATURE REVIEW

2.1 Mouse and human embryonic stem cell cultures

The techniques to initiate primary cultures from the inner cell mass (ICM) of preimplantation blastula stage embryos of mouse were first established in the early 1980s (Axelrod, 1984; Doetschman *et al*., 1985; Evans & Kaufman, 1981; Martin, 1981; Wobus *et al*., 1984). Initially, mES cells were maintained on feeder layers which consisted of chemically inactivated mouse embryonic fibroblast (MEF) cells. Although the capability of MEF feeder cells in supporting ES cells were poorly defined, it was believed to promote self-renewal or reduce differentiation. It was found that mES cells can be cultured in the absence of feeder layers if leukemia inhibitory factor (LIF) was added into the culture medium (Smith *et al*., 1988; Williams *et al*., 1988). Established mES cells showed unlimited proliferation capacity *in vitro*, retained normal and stable chromosome numbers as well as retained the ability to differentiate into cell types from the three germ layers (Evans & Kaufman, 1981; Martin, 1981). mES cells also showed a comparatively short generation time of around 12 to 15 hours and they had a short G_1 cell cycle phase (Rohwedel *et al*., 1996).

There are several well established criteria that are commonly used to characterize mES cells. It is well-known that mES cells express membrane-bound receptors (gp130) (Niwa *et al*., 1998), alkaline phosphatase (Wobus *et al*., 1984) and telomerase activities (Armstrong *et al*., 2000). Telomerase is a reverse transcriptase

encoded by the telomerase reverse transcriptase (*Tert*) and telomerase RNA component (*Terc*) genes, which functions to add telomeric repeats onto chromosome ends to provide chromosomal stability (Blackburn, 2005; Collins & Mitchell, 2002). In ES cells, telomere length and cellular immortality are maintained by high telomerase activities (Hiyama & Hiyama, 2007). Oct-3/4 is also found to be crucial in mES cells as it is needed at suitable level to maintain pluripotency. Increase in expression of Oct-3/4 level by less than twofold will cause differentiation into primitive endoderm and mesoderm, while inhibition of Oct-3/4 expression will cause the cells to differentiate into trophectoderm and lose pluripotency ability (Niwa *et al*., 2000a). Nanog has also been identified as an additional key regulator of mES cells pluripotency (Chambers *et al*., 2003; Mitsui *et al*., 2003). Among other molecular markers that are used to characterize mES cells are Rex-1 (Rogers *et al*., 1991), Sox2 (Avilion *et al*., 2003), Genesis (Sutton *et al*., 1996), UTF1 (Okuda *et al*., 1998) and GBX2 (Chapman *et al*., 1997). The functions of these molecular markers in controlling self-renewal remain to be determined, as they are not exclusively expressed by the ES cells only, they are also expressed by other somatic cells, even though they are downregulated during differentiation of ES cells (Wobus & Boheler, 2005).

The successful establishment of mES cells has led to the derivation of hES cells from human blastocysts (Pera *et al*., 2000; Reubinoff *et al*., 2000; Stojkovic *et al*., 2004; Thomson *et al*., 1998). hES cells share several similar characteristics with mES cells such as expression of Oct-3/4, alkaline phosphatase activity, telomerase activity, teratoma formation *in vivo*, prolonged culture periods and maintenance of stable chromosome numbers (Amit *et al*., 2000; Richards *et al*., 2002; Thomson *et al*., 1998). However, unlike mES cells, hES cells do not respond to LIF as it fails to

inhibit differentiation of hES cells sufficiently (Reubinoff *et al*., 2000; Thomson *et al*., 1998). Therefore, hES cells are normally maintained on feeder layers which consist of MEF cells or human feeder cells, although the properties of these feeder layers in supporting hES cells remain poorly understood. Similar to mES cells, hES cells are now maintained using human feeder cells or in feeder-free system under serum-free medium formulations in order to prevent xenogenic contaminations (Amit *et al*., 2003; Lee *et al*., 2005; Richards *et al*., 2002; Ying *et al*., 2003). These developments will increase the potentials of utilizing pluripotent ES cells in therapeutic applications and regenerative medicines as well as providing key insights for researchers to further understand developmental biology.

One ES cell line is usually not the same as other ES cell lines in terms of morphologies, growth characteristics, differentiation potentials, molecular signatures and culturing techniques. Therefore, it is difficult to apply a common standard for characterization of all human and mouse ES cell lines (Smith *et al*., 2009). Establishment of a stringent set of molecular, cellular, functional and developmental pluripotency tests is crucial in defining the undifferentiated and differentiated state of human and mouse ES cells (Smith *et al*., 2009). In addition, transcriptome profiling and proteomic analyses should be carried out together during characterization of ES cell lines as these information will be essential for creating standards in mouse and human ES cell research such as defining the optimal growth conditions as well as determining which ES cell lines are suitable for downstream applications (Brivanlou *et al*., 2003).

2.2 Embryonic stem cell cultures from zebrafish

Due to the successful establishment of mouse and human ES cells, works on establishing fish ES cell lines have been carried out since 20 years ago by using the feeder layer or feeder layer-free culture conditions in zebrafish (Collodi *et al*., 1992; Sun *et al*., 1995b) and medakafish (Hong *et al*., 1996; Wakamatsu *et al*., 1994). There are also several fish ES cell lines established from marine fish *Sparus aurata* (Béjar *et al*., 2002), red sea bream *Chrysophrys major* (Chen *et al*., 2003a), sea perch *Lateolabrax japonicas* (Chen *et al*., 2003b), marine flatfish *Scophtalmus maximus* (Holen & Hamre, 2003), Asian sea bass *Lates calcarifer* (Parameswaran *et al*., 2007), Atlantic cod *Gadus morhua* (Holen *et al*., 2010) and Indian major carp *Catla catla* (Dash *et al*., 2010). For most of these fish species, characterizations on the ES cell lines were carried out based on their cell morphologies, alkaline phophatase activities, immunocytochemistry analysis and chimera formation abilities.

In zebrafish, the culture of ES cells on feeder layer has only produced partial success and long-term culture of zebrafish ES cells on feeder layer has not been established (Collodi *et al*., 1992; Sun *et al*., 1995b). For feeder layer culture system, a confluent monolayer of growth-arrested rainbow trout spleen cells (RTS34st) was used to initiate zebrafish embryo cell culture from blastula and gastrula stage embryos (Collodi *et al*., 1992; Sun *et al*., 1995b). Although poorly defined, it is believed that the RTS34st cells provide some factors that help to maintain the zebrafish cultures in a germ-line competent state (Fan & Collodi, 2004). Germ-line chimeras have been obtained from short-term culture of zebrafish embryo cells on growth-arrested RTS34st cells (Fan *et al*., 2004b; Ganassin & Bols, 1999; Ma *et al*., 2001). It has also been observed that zebrafish ES cells are able to contribute to

multiple tissues including germ cell lineage of the host upon microinjection into a recipient embryo (Fan & Collodi, 2002).

The culture medium used for derivation of zebrafish ES cells in feeder layer system by Fan and Collodi, (2004) includes LDF (a mixture of Leibovitz's L-15, Dulbecco's modified Eagle's and Ham's F-12 media) as the basal medium, supplemented with fetal bovine serum (FBS), trout serum, bovine insulin, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), zebrafish embryo extracts and RTS34st cell-conditioned medium. These culture conditions enabled the culture of zebrafish germ-line competent ES cells for at least six passages or six weeks (Fan & Collodi, 2004). The zebrafish ES cells formed dense and homogenous aggregates without showing signs of differentiation. This enables electroporation and selection of homologous recombinants to be carried out with sufficient time (Fan *et al*., 2004a). However, there are some drawbacks in the culture medium used as it contains several undefined components. Furthermore, the presence of RTS34st feeder layer has many disadvantages such as fluctuation of culture conditions depending on feeder cells, possibility of contamination from feeder cells and sub-culturing limits due to short life span of feeder cells (Hong *et al*., 1996; Xing *et al*., 2008).

In order to overcome drawbacks from the feeder layer system, works on the establishment of zebrafish ES cells in feeder-free conditions have been carried out about six years ago by Xing *et al*., (2008). In this study, zebrafish blastula cells were initiated on RTS34st feeder layer and subsequently transferred into a feeder-free condition, using a simpler culture medium containing L-15 and FBS. The zebrafish blastula cell line was designated as ZEB2J. However, it has limited differentiation

potential when it is cultured in the absence of feeder layer (Xing *et al*., 2008). More recently, a study on transient feeder-free zebrafish ES cell cultures was carried out by Robles *et al.,* (2011). One of the contributions of this study is the usage of a completely defined culture medium which consisted of LDF basal medium supplemented with B27 and N2 serum-free supplements with two small molecule inhibitors, glycogen synthase kinase 3 (GSK3; CHIR99021) and mitogen-activated protein kinase 1 (MEK-1; PD184352) inhibitors. It was found that MEK-1 inhibitor prevented differentiation of the ES cells, but it affected the growth and viability of cells. As such, GSK3 inhibitor was added to provide a better condition for self-renewal of the ES cells (Robles *et al.,* 2011). Although several insights into the pluripotency-related markers were shown in this study, long-term and stable feeder-free zebrafish ES cell cultures have not been established.

It was found that proper cell density and culture medium are required to successfully establish an ES cell line (Hong *et al*., 1996). This is to ensure that developing fish ES cells have an essential environment for them to grow without undergoing spontaneous differentiation. Several studies on medakafish and zebrafish have found out that fish embryo extracts and bFGF are required for mitogenic activity of the ES cells (Collodi *et al*., 1992; Hong & Schartl 1996; Sun *et al*., 1995a; Sun *et al*., 1995b; Wakamatsu *et al*., 1994). In both fish species, LIF is found to have no effect on cell growth and differentiation (Hong & Schartl, 1996). The ability of fish ES cells to be independent of feeder layer and LIF provides a crucial advantage over other non-piscine species, in terms of manipulation of the ES cells (Alvarez *et al*., 2007). Finally, it is important to identify cellular and molecular genetic markers that control self-renewal and differentiation, as well as *in vitro* and *in vivo* characterization of fish ES cell lines, so that more in-depth understanding and

insights into early development in fish species and the potential of fish ES cells can be fully explored (Alvarez *et al*., 2007).

2.3 Zebrafish as a model organism

Emergence of zebrafish as a major model organism for biological research was first initiated by the late George Streisinger and colleagues at the University of Oregon (Streisinger *et al*., 1981). Zebrafish has been highly valued as a model organism for developmental biology (Weis, 1968), toxicology (Hisaoka, 1958) and genetic (Kimmel, 1989) research. Among the advantages of using zebrafish as a model organism are the transparency of the embryos and external development which enable easy observations of developing anatomical structures under the microscope (Eisen, 1996). This also enables in-depth studies on cell fate and patterning (Schier, 2001). However, the highest contribution of zebrafish model in the analysis of intricate biological processes such as developmental biology and aging remains in the capability to manipulate it genetically (Talbot & Hopkins, 2000).

Due to the short generation times of zebrafish, thousands of fish can be produced conveniently and large populations can be sustained economically in the laboratory (Gerhard & Cheng, 2002). This is because one female zebrafish can produce up to several hundreds of eggs per week (Gerhard & Cheng, 2002). High productivity of zebrafish enables the identification of developmentally significant genes to be carried out through various large-scale mutagenesis screens in zebrafish (Ma *et al*., 2001). In fact, the first large-scale mutagenesis screens of zebrafish were carried out by Nüsslein-Volhard and colleagues in Germany in early 1990s (Haffter *et al*., 1996) to identify developmental mutations (Gerhard & Cheng, 2002). The

screens were carried out based on her Nobel prize-winning studies on saturation mutagenesis screens in *Drosophila* (Nüsslein-Volhard & Wieschaus, 1980) and studies carried out by Fishman and colleagues in Boston (Gerhard & Cheng, 2002, Stainier *et al*., 1996).

Various studies on zebrafish can be done as there are abundant genomic resources available such as databases of expressed sequence tags, high-density genetic linkage, radiation hybrid maps and a continuous effort to sequence the zebrafish genome (Amemiya *et al*., 1999; Clark *et al*., 2001; Hukriede *et al*., 1999; Shimoda *et al*., 1999; Vogel, 2000). These vast resources have placed zebrafish in an elite group of model organisms together with *Caenorhabditis elegans, Drosophila melanogaster* and *Mus musculus* (Gerhard & Cheng, 2002). Toxicological and pharmacological studies can also be done using minimum quantities of chemicals and the amounts of potentially hazardous waste can be reduced through a miniaturized format provided by the tiny sized larval and adult zebrafish (Hill *et al*., 2005; Spitsbergen & Kent, 2003).

In addition, sufficient databases can be obtained from numerous replicate samples for statistical analysis and validation of results as the eggs and juveniles are small in size and high-throughput screenings can be done in multi-well culture plates (Bopp *et al*., 2006). Another crucial characteristic of zebrafish is that mutant zebrafish embryos are able to survive substantially through the time in which the particular organs usually begin to function in healthy individual whereas malformed embryos of rodents usually die in the uterus (Hill *et al*., 2005). Mutants are very important for various studies such as human diseases. Hundreds of zebrafish phenotypic mutants were created and utilized to resolve molecular regulations of

ontogeny (Stern & Zon, 2003). All these advantages, combined with the availability of various new technologies as well as the spreading of their applications in numerous fields have made the zebrafish uniquely matched to revealing the genetic regulatory networks that bring about the development and function of vertebrates (Carpio & Estrada, 2006).

2.4 Zebrafish

The natural habitat of zebrafish is in slow streams, rice fields and in the Ganges region of northern India as well as in Pakistan, Bangladesh, Nepal and Myanmar (Mayden *et al*., 2007; Menon, 1999; Spence *et al*., 2008; Talwar & Jhingran, 1991). The scientific name for zebrafish is *Danio rerio*. One of the unique characteristics that distinguished zebrafish from other fish species is the patterning of its body. An adult zebrafish has five uniform and horizontal dark blue stripes on the sides of its body. The size of an adult zebrafish is only approximately 3 cm to 5 cm in length. Thus, they can be easily maintained in large quantity in the laboratory (Kishi *et al*., 2003). Zebrafish was first described by the British surgeon, Francis Hamilton in 1822 (Quigley & Parichy, 2002). It is categorized under the family of the cyprinids (Cyprinidae) in the class of ray-finned fishes (Actinopterygii) and within this class, to the bony fishes known as Teleostei or teleosts (Carpio & Estrada, 2006). Adult male and female wild-type zebrafish are shown in Figure 2.1.

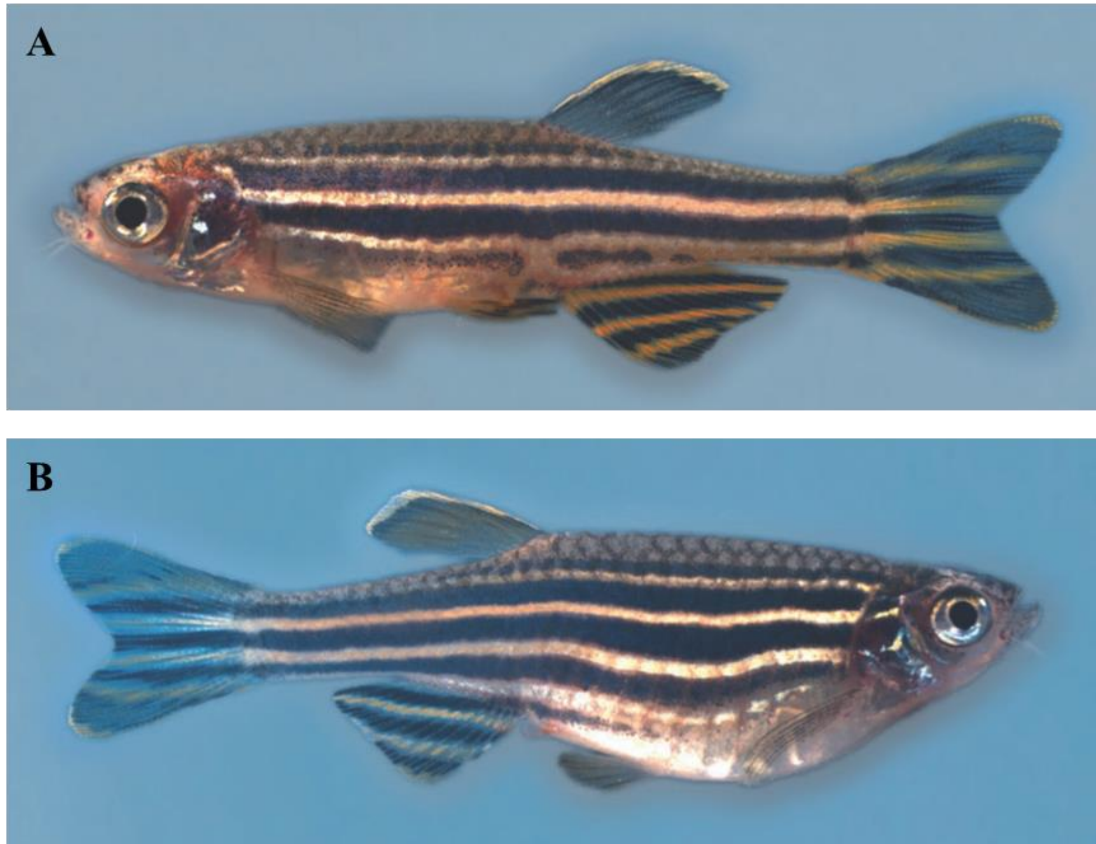

Figure 2.1: Wild-type zebrafish (*Danio rerio*). (**A**) Adult male zebrafish and (**B**) adult female zebrafish (adapted from Gomez de la Torre Canny *et al*., 2009).

Generally, zebrafish can be maintained using tap water which has been conditioned by letting it set and equipped with biological filters. Alternatively, the tap water can also be treated through reverse osmosis in order to remove excessive chlorine, chloramines, copper, nitrogen, phosphates, silicates or other chemicals which might affect or harm the fish. Approximately 25 fish can be reared in a 45 liter aquarium set at 28.5°C (Westerfield, 2007). A 14-hour light and 10-hour dark cycle will be essential for maintaining zebrafish (Brand *et al*., 2002). The pH of the water should be around pH 6.0 to pH 8.0 with water hardness of 5.0 to 19.0 degrees of general hardness (dGH) (Kishi *et al*., 2003). Zebrafish can be fed with commercially

available fish flakes, frozen or live brine shrimps, live tubifex worms, cichlid pellets and hard-boiled egg yolks.

Zebrafish embryogenesis is very similar to higher vertebrates such as humans. Many human developmental and disease genes have counterparts in the zebrafish as there are extensive similarities between the zebrafish and human genomes (Howe *et al*., 2013). However, zebrafish has short generation times of only about three to five months (Bopp *et al*., 2006). Zebrafish embryos develop externally as the fertilized eggs develop into an adult outside the female. Zebrafish embryos are transparent during the first few days of their lives (Wixon, 2000). Zebrafish has very fast embryonic development as in the first 24 hours post-fertilization (hpf), all major organs are already developed even though the early larval pigment patterns are not fully developed until a few days later (Wixon, 2000). The fish will hatch and start looking for food within three days. Zebrafish will be sexually matured and ready to produce offspring after three to four months. One female zebrafish can lay almost 700 eggs per week (Spence & Smith, 2006). The average lifespan of zebrafish is 3.5 years, even though some can survive for up to 5.5 years (Gerhard *et al*., 2002).

2.5 Characterization of embryonic stem cells

Characterization of a cell line is important before it can be classified as an ES cell line. Among the criteria involved are morphological comparisons with other ES cell lines, unlimited proliferation potential *in vitro*, ability to remain undifferentiated while maintaining potential to differentiate into cell types from three germ layers, expression of specific pluripotency-related markers, maintenance of stable chromosome numbers and capacity to produce germ-line chimeras (Smith *et al*.,

2009). The three germ layers consisted of ectoderm, endoderm and mesoderm, where various organs and parts of an organism develop through further differentiation during embryogenesis. Pluripotency is a unique characteristic of ES cells which are obtained from the ICM of a blastula embryo (Smith *et al*., 2009). Unlike totipotent cells which are the most versatile stem cells and can give rise to an entire functional organism, pluripotent cells do not form the trophectoderm. The trophectoderm is the outer layer of cells which is important for successful embryonic development as it is required for the formation of extraembryonic structures like the placenta (Smith *et al*., 2009). As the pluripotent state is extremely dynamic and it might produce variable results in molecular studies, development of a stringent set of criteria to determine pluripotency is very valuable in ES cell research (Smith *et al*., 2009).

2.5.1 Morphology, cell cycle and clonal selection

Mouse and human ES cells have distinguishable morphology, as they normally grow in compact and round shaped colonies with smooth edges (Evans & Kaufman, 1981; Thomson *et al*., 1998). There are two morphological characteristics of ES cells – they have large nuclei and sparse cytoplasm. They also have faster proliferation rate compared to other cell types (Kumar *et al*., 2009). However, not all ES cells have the same morphology, as ES cells from other mammalian species have been found to appear as a more flattened monolayer of colonies with individually distinct cells that have epithelial-like morphology (Familari & Selwood, 2006) or dome shaped colonies with plentiful lipid-like vacuoles (Verma *et al*., 2007). For fish species like the medakafish, the ES cells are usually round or polygonal in shape with high nucleus to cytoplasm ratio and prominent nucleoli (Hong *et al*., 2011). The

fast proliferation rate of ES cells is characterized by a shortened G_1 phase during mitosis cycle (Becker *et al.*, 2006; Ghule *et al.*, 2007; Ghule *et al.*, 2008). In addition, ES cells also have a unique nuclear structural design including lamina, nuclear speckles, heterochromatin domains and chromatin structures (Meshorer & Misteli, 2006).

Similar to human and mouse ES cells, fish species ES cells are also capable of forming colonies and clonal cultures can be carried out to screen for pluripotency *in vitro* and *in vivo* (Hong *et al.*, 1996; Béjar *et al.*, 2002). As ES cells are isolated from the ICM of blastocysts, the cell populations used to establish ES cell cultures should therefore be considered as heterogeneous (Amit *et al.*, 2000; Heins *et al.*, 2006). Homogeneous ES cell cultures could be obtained through subcloning or clonal selection (Amit *et al.*, 2000; Heins *et al.*, 2006). Most of hES cell lines have not undergone clonal selection, thus it could not be ruled out that they are mixtures of multiple precursor cells which are already committed to certain cell lineages (Heins *et al.*, 2006), even though majority of reported hES cells showed homogenous appearances with similar morphologies and specific hES markers (Carpenter *et al.*, 2003). Attempts to derive clonal sublines from parental hES cell lines in order to further enhance hES cell populations were successfully carried out by Amit *et al.*, (2000) and Heins *et al.*, (2004).

Clonal selection is essential in selecting subclones of ES cells which consist of homogenous diploid population of undifferentiated cells, as it has been shown that ES cells are susceptible to karyotypic aberrations under particular culture conditions (Draper *et al.*, 2004b; Inzunza *et al.*, 2004; Mitalipova *et al.*, 2005; Rosler *et al.*, 2004). Clonal selection also played a significant role when ES cells with identical

genetic composition are needed for various high throughput applications and transcriptional profiling (Fuchs *et al*., 2004; Heins *et al*., 2006). As ES cells are very sensitive to their microenvironment, the task of defining a specific molecular signature for a particular ES cell line would be very challenging, emphasizing the necessity to isolate ES cell subclones from their parental lines (Fuchs *et al*., 2004). It would be a matter of the utmost importance to isolate and evaluate cells in ES state and their immediate progenies in order to identify the key differences in transcriptional profiling between ES cells and cells that have just committed to a certain fate (Fuchs *et al*., 2004).

2.5.2 Cytogenetic analysis

Another important characterization of an ES cell line is chromosome counts or analysis, as an ES cell line should possess normal diploid chromosome numbers so that it can contribute to germ-line chimera (Alvarez *et al*., 2007). Tubulin inhibitor like colchicine is commonly used to depolymerize mitotic spindle of cells in order to arrest them during mitosis for chromosome banding (Bickmore, 2001). The usage of fluorescent dyes or fluorochromes that bind to AT-rich DNA such as 2-(4-amidinophenyl)-1H -indole-6-carboxamidine (DAPI) in chromosome banding has become increasingly popular (Bickmore, 2001). DAPI has advantages over other fluorochromes as it is highly resistant to fading and its wavelengths are compatible with filters commonly used in fluorescent imaging (Bickmore, 2001). The banded chromosomes are portrayed in a typical format called karyogram, where the chromosomes will be arranged in pairs of equal sizes and position of centromeres (Gustashaw, 1991).

Several studies on chromosome numbers for long-term ES cell cultures have been reported. hES cells cultured for extended passage numbers ranging from passage 24 to 140 has been reported to be able to retain normal chromosome numbers (Buzzard *et al*., 2004). Other than hES cell lines, ES cell lines from other species such as mouse, buffalo, bovine and sheep have also been reported to maintain normal chromosome numbers at different passage numbers (Dattena *et al*., 2006; Evans & Kaufman, 1981; Strelchenko *et al*., 1995; Verma *et al*., 2007). In addition, the chromosome numbers of ES cell lines from several fish species such as Asian sea bass, medakafish, sea perch, Indian major carp and red sea bream have also been shown to be stable after multiple passages (Chen *et al*., 2003a; Chen *et al*., 2003b; Dash *et al*., 2010; Hong *et al*., 1996; Parameswaran *et al*., 2007).

2.5.3 Expression of various cell markers

2.5.3.1 Alkaline phosphatase

Alkaline phosphatase (AP) enzyme is produced by nearly all cell types, but its activity is the highest in ES cells (Kumar *et al*., 2009). AP is found on the cell surface as it is linked to the cell membrane through a phosphatidylinositol glycan linkage (Kumar *et al*., 2009). The true function of AP during physiological conditions remains largely unidentified, although it is classified base on its ability to hydrolyze orthophosphate monoesters at alkaline pH (Kumar *et al*., 2009). AP activity is an important marker for pluripotency as it is reduced or lost upon differentiation. Therefore, AP activity is commonly monitored in mES cells (Piquet-Pellorce *et al*., 1994; Wobus *et al*., 1984; Yoshida *et al*., 1994). Various reports have shown that hES cells display AP activity too (Carpenter *et al*., 2003; Ginis *et al*.,

2004; Smith, 2001). High AP activity is also found in ES cells from medakafish using the feeder layer and feeder layer-free culture systems (Hong & Schartl, 1996; Wakamatsu *et al*., 1994).

2.5.3.2 Immunocytochemistry analysis

Immunocytochemistry is the *in situ* identification of a specific tissue component by using specific antigen-antibody interaction which involves tagging of the antibody with a certain visible dye (VanNoorden & Polak, 1983). The use of specific antibodies to stain cell structures is an extremely powerful method as it shows both the presence and subcellular localization of a distinct molecule of interest (Sternberger, 1979). The very first attempt to use normal dyes to label antibodies was not successful as the normal dyes are not easily viewable under the microscope (Mao *et al*., 1999). Immunocytochemistry was improved by Coons *et al*., (1941) when fluorescent dyes were used to label specific antibodies to view the localization of a particular molecule of interest in tissues. However, the potential of this technique was not realized until 20 years later as many claimed it to be tedious (Mao *et al*., 1999).

Initially, direct immunocytochemistry was applied, where a specific antibody itself was directly labeled with fluorophore (Coons *et al*., 1941). Later on, a more precise and flexible method known as the indirect immunocytochemistry was applied (Coons *et al*., 1955). According to Kumar *et al*., (2009), indirect immunocytochemistry involves detection using a secondary antibody tagged with either a fluorophore or an enzyme. The tagged secondary antibody will then bind to a specific primary antibody which is bound to the antigens of the cells. The ability of

specific primary antibodies to recognize antigens on the surface or intracellular structures of cells is greatly valued in characterization of cell types (Kumar *et al.*, 2009). Cell surface and intracellular antigens offer an invaluable means in the analysis and cell sorting fields as it enables a researcher to understand further on the particular characteristics within specific contexts (Kumar *et al.*, 2009).

2.5.4 Differentiation potentials

One of the characteristics of an ES cell line is pluripotency, which is the ability to differentiate into cell types from the three germ layers *in vitro* and *in vivo* (Smith *et al.*, 2009). The ultimate proof of pluripotency for any ES cell line would be the ability to form germ-line chimera when the cells are injected back into the host embryo (Smith *et al.*, 2009). Formation of embryoid bodies (EB) when they are cultured in suspension is also one of the differentiation potentials of ES cells. These EB are able to form cell lineages of all three germ layers (Itskovitz-Eldor *et al.*, 2000). ES cells are able to differentiate *in vitro* into various cell types in culture grown at different densities or be induced to differentiate by certain chemicals or small molecules. When all-*trans*-retinoic acid is added into the culture medium, ES cells from several fish species can be induced to differentiate into epithelial, fibroblast or neuronal-like cells (Béjar *et al.*, 2002; Chen *et al.*, 2003a; Chen *et al.*, 2003b; Hong *et al.*, 1996; Sun *et al.*, 1995b; Wakamatsu *et al.*, 1994). *In vitro* differentiations at low and high densities have also induced fish ES cells to differentiate into cell types from the three germ layers (Chen *et al.*, 2003a; Chen *et al.*, 2003b; Hong *et al.*, 1996). For hES cells, it has been found that high density cultures for extended periods of four to seven weeks without replacement of a feeder

layer had supported differentiation into multicellular aggregates or vesicle structures that formed on top of the monolayer of cells, as well as clusters of cells or single cells with elongated processes coming out from their cell bodies (Reubinoff *et al.*, 2000).

2.6 Application and future prospects of zebrafish embryonic stem cells

2.6.1 Gene targeting

As early as 1987, studies have shown that DNA could be transfected into the mES cell genome through homologous recombination (Thomas & Capecchi, 1987). Two years later, the first report on germ-line transmission of a targeted genetic modification in mES cells that contributed to viable chimera formations was published (Thompson *et al.*, 1989). Today, gene targeting is the most powerful tool used in many laboratories to introduce defined genetic modifications into the genome of ES cells (Bradley *et al.*, 1992; Joyner, 1991). Gene targeting offers an opportunity to elucidate the physiological functions of certain genes in question, but not every biological process can be examined through gene inactivation (Wobus & Boheler, 2005). In some cases, gene targeting causes developmental arrest or embryonic lethality. This reveals the dependency of an embryo on a certain gene for its earliest development and rules out analysis of the function of a certain gene at later developmental stages (Wobus & Boheler, 2005). These drawbacks can be overcome by producing conditional knock-out or knock-in ES cells and mice, where a particular gene of interest can be spatially and also temporally activated or inactivated (Nagy, 2000).

In fish ES cell technology, ES cells were initially developed as a means to produce transgenic fish to solve basic biological problems or to enhance the productivity in commercially farmed fish species (Alvarez *et al*., 2007). Even though gene targeting is a highly valuable technique, the success rate of precise gene targeting is rare when compared to random integration (Hong *et al*., 2011). This, however, can be overcome through a procedure known as positive-negative selection (PNS), where enhancement of homologous recombinants in mES cells is done by using drug selection that helps to eliminate random events to a great extent (Mansour *et al*., 1988). A study has shown that selectable marker genes that were used in mouse can also be applied in the carp cell line, EPC (Chen *et al*., 2002). This shows the potential application of gene targeting in fish species. One of the remarkable achievements of gene targeting in fish ES cells is the MES1 cell line, where the conditions for gene transfer and drug selection have been optimized to serve as preliminary guidelines for gene targeting in fish (Hong *et al*., 2004).

It would be highly valuable to establish gene targeting techniques for the production of knock-out and knock-in lines of zebrafish in order to fully utilize it as a model organism to study gene function (Fan & Collodi, 2006). As a complement to transient antisense-based gene knockdown strategies, knock-out and knock-in techniques using targeted insertional mutagenesis enable the characterization and functional study of a particular gene of interest during later developmental stages and even in adult fish (Fan & Collodi, 2006). Knock-out studies have been carried out in mice for more than two decades, where vector DNA is inserted into a specific locus in mES cells through homologous recombination (Capecchi, 1989; Doetschman *et al*., 1987). Colonies of mES cells containing the targeted insertion are selected and expanded before they are injected into host embryos to contribute to germ-line

chimeras. Knock-out lines in mice are then produced by germ-line chimeras that carry the targeted insertion (Bradley *et al*., 1992; Joyner, 1991). So far, knock-out and knock-in strategies have not been successful in other species, including zebrafish, due to the lack of real ES cell lines that are capable of producing germ-line chimeras and lack of knowledge on zebrafish pluripotency-related markers (Fan & Collodi, 2006; Robles *et al*., 2011).

Fan *et al*., (2006) have demonstrated that plasmid can be incorporated into zebrafish ES cells via homologous recombination by targeting the inactivation of two genes, *no tail* (*ntl*) and *myostatin 1* (*mstn 1*). In this study, PNS strategy was used to select colonies of cells with the targeted insertion (Fan *et al*., 2006). The first selection involved a bacterial selectable marker gene, *neo* which is located inside a region of the vector that is homologous to the gene of interest and a red fluorescent protein gene (RFP) which is located outside of the homologous region (Fan *et al*., 2006). The plasmid was introduced into zebrafish ES cells through electroporation and transfected cells were subsequently selected by using geneticin (G418) (Fan *et al*., 2006). All of these show that gene targeting is possible in zebrafish. Nevertheless, a lot of work still need to be done to obtain pure targeted ES clones that remain pluripotent and capable of achieving germ-line transmission (McCarrick *et al*., 1993).

2.6.2 Directed differentiation and transplantation biology

mES cells have been used as a valuable tool to study differentiation processes *in vivo* (Donovan & Gearhart, 2001). However, the potential of ES cells to act as differentiation models has not been fully developed yet as efforts to obtain homogenous ES cell differentiation are still in their infancy (Yi *et al*., 2010b).

Availability of homogenous populations of specialized cells and ability of these cells to be incorporated into the patients' physiological conditions after transplantation are crucial for stem cell-based therapy (Yi *et al*., 2010b). An ES cell line from medakafish, MES1, has been shown to be able to undergo directed differentiation into melanocytes by transfecting the cells with melanocyte-specific isoform of the *microphtalmia transcription factor* (*mitf*) (Yi *et al*., 2010b). The success of directed differentiation in MES1 has shown that proper differentiation of fish ES cells can be done and fish ES cells can be suitable differentiation models as well. As of now, directed differentiation experiments have not been carried out in zebrafish, due to the lack of long-term and stable zebrafish ES cell lines.

Zebrafish eggs develop rapidly as transparent embryos which enable the visualization of all stages of vertebrate development to be done effortlessly (Wixon, 2000). Differences in gene expression and thorough morphogenetic movements as they occur in a live, developing vertebrate embryo can be visualized when combined with fluorescent reporter genes (Udvadia & Linney, 2003). A major contribution of zebrafish in vertebrate developmental studies is the opportunity to carry out real-time imaging in the natural environment of the developing embryo (Udvadia & Linney, 2003). The normal opacification of skin and subdermal structures of adult zebrafish has limited analysis to be done on embryonic development stages only even though zebrafish is a useful model organism for the study of normal and cancer stem cells (White *et al*., 2008). The development of a transparent adult zebrafish model by White *et al*., (2008) has enabled the complex behavior of stem cell populations to be examined *in vivo*, in addition to *in vitro* models. Full characterization on the dynamics and spatial characteristics of stem cell transplantation can be studied at the